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13. ABSTRACT ***(Maximum 200 Words)***

The studies in this proposal sought to define mechanisms by which the adenoviral E1A oncogene induces the phenotypic conversion of human tumor cells from injury-resistant to injury-sensitive in the context of immunological and chemotherapeutic antineoplastic agents. Basic observations using NIH-3T3 cells were used to model translational studies in human breast cancer cells and other types of human tumor cells. The studies were divided into two tasks: (1) Definition of molecular pathways through which E1A triggers cellular sensitivity to injury and (2) Analysis of the mechanisms by which E1A expression represses cellular defenses against apoptosis. In the first task, studies showed that E1A-induced phenotypic conversion to injury-sensitive was mediated through apoptotic, and not necrotic, cell death pathways and that p53 tumor suppressor activity was not required. These data were complemented by studies of E1A-induced differences in cellular gene expression. E1A-induced apoptosis sensitivity was not blocked the the Bcl-2 like activity of the E1B 19 kD gene. These in vitro observations were used as the basis for studies of E1A effects on human tumor cell susceptibility to rejection by host innate immune defenses and experimental chemotherapy.

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INTRODUCTION:

These studies are based upon our observation that the adenoviral E1A oncogene can convert highly cytolytic-resistant human tumor cells into cells that are highly susceptible to cytolysis triggered by a variety of immunological and chemical injuries. Observations made using mouse NIH-3T3 cells were translated to human breast cancer cells and other types of human tumor cells. This comparative analysis revealed that there are basic cellular traits controlled by E1A that are common among species and diverse cell types. Studies of the regulatory control pathways through which E1A-induced phenotypic conversion of human tumor cells is mediated were complemented by studies of differential gene expression. Studies of these molecular mechanisms were used as a basis for comparisons with in vivo studies of innate immunity-mediated and chemotherapy-induced rejection of E1A-expressing human tumor cells in vivo.

BODY:

Task one was to identify cellular genes and pathways whose modulation by E1A is involved in increased tumor cell susceptibility to injury-induced cell death response. Initial studies confirmed the hypothesis that E1A expression renders tumor cells susceptible to apoptotic cell death but does not specifically sensitize cells to death by necrosis [1]. This conclusion resulted from studies of both immunological and chemical injuries of paired E1A-expressing an E1A-negative cell lines.

In addition to identifying general mechanisms of this E1A-induced control of cellular phenotype, Task one was used as the focus for studies of differential gene expression in E1A-positive versus E1A-negative cells. The purpose was to develop a library of information that could be used to identify for future experiments candidate genes whose expression might be targeted by E1A to change the apoptosis phenotype from resistant to sensitive. These studies were started using the method of differential display but, because of the greatly increased reporting capabilities of cDNA array analysis, were converted to studies using the Affymetrix cDNA array system. These experiments have been completed and validated for E1A-related changes in expression of genes from several different functional categories. These results open numerous new questions about control mechanisms through which E1A oncogene expression sensitizes cells to apoptotic injury. The relevance of these observations will be tested using northern analysis and biological assays to test hypotheses regarding the roles of selected genes in cellular sensitization to injury.

An example of the hypothesis generating capability of this analysis of E1A-induced differential gene expression is our observation that E1A represses expression of cellular osteopontin (OPN). OPN is a secreted phosphoprotein of the integrin superfamily that is the ligand for the vitronectin receptor (alpha v beta 3). Through this interaction, OPN can repress expression of inducible nitric oxide synthase (iNOS) [2]. OPN is inducible in various cell types exposed to cytokines, including IL-1 and TNF alpha [3, 4]. Through its iNOS suppressive activity, OPN can reduce cellular susceptibility to cytotoxic cells and

their mediators that would otherwise induce apoptosis in tumor cells [2]. It is relevant for this proposal that over 80% of invasive breast cancer lesions overexpress OPN [5]. It has been postulated that these effects explain the correlation between high level OPN expression and the metastatic potential of breast cancer cells [6]. In our studies, OPN was repressed eightfold by E1A expression compared with OPN expression in E1A-negative cells. This observation suggests the hypothesis that E1A represses tumor cell secretion of OPN, thereby reducing the tumor cell defenses against macrophage cytotoxicity. We are testing the role of regulated OPN expression in the resistance and E1A-induced susceptibility of breast cancer and other cell types to immunological and chemotherapy-induced injury.

Task two was to begin to test the cellular apoptosis pathways involved in E1A enhancement of the cytolytic and apoptotic responses of breast cancer cells. We have asked two questions to focus these studies. First, are expression of the p53 tumor suppressor gene and its regulated pathways necessary for E1A-induced cellular sensitivity to injury-induced apoptosis? Second, is E1A induced apoptosis sensitization blocked by the antiapoptotic effect of Bcl-2-family proteins?

Both rodent cells and human cells with loss-of-function mutations in p53 or completely lacking p53 expression were sensitized to proapoptotic injuries by E1A as well as cells that expressed native p53 [7]. This conclusion was supported by studies of p53-mutant human fibrosarcoma and melanoma cells, both of which were converted to apoptosis-sensitivity by E1A expression. These observations indicate that there are p53-independent mechanisms by which E1A can induce phenotypic conversion of tumor cells to apoptosis sensitivity and suggest the possibility that these p53-independent mechanisms exist latently in all types of human tumor cells.

We used the adenoviral E1B 19 kD gene to test the effect of Bcl-2-like antiapoptotic activities on E1A induced apoptosis sensitivity. E1B 19 kD is an antiapoptotic member of the Bcl-2 family. We had reported that E1B 19 kD expression does not prevent E1A induction of cellular susceptibility to lysis by killer lymphocytes or activated macrophages [8]. Studies were done to test the prediction the E1B 19 kD also would not block TNF-induced apoptosis and to contrast the E1B 19 kD effects on immune-mediated apoptosis with effects against various proapoptotic, chemical injuries [7]. E1A-positive cells with or without expression of E1B 19 kD were tested for sensitivity to killer lymphocytes, TNF alpha, hygromycin (a protein synthesis inhibitor), beauvericin (a potassium ionophore), etoposide (a topoisomerase II inhibitor) and hydrogen peroxide. E1B 19 kD did not block E1A-induced sensitivity to killer lymphocytes or TNF alpha, but E1B 19 kD did block E1A-induced sensitization to apoptosis triggered by hygromycin, beauvericin, etoposide and hydrogen peroxide. The ability of E1B 19 kD to block E1A-induced sensitization apoptosis was not linked to the p53-dependence of the proapoptotic injury, since hygromycin (a p53-independent injury) and all three p53-dependent injuries (beauvericin, etoposide and hydrogen peroxide) were blocked by E1B 19 kD, but killer lymphocytes and TNF alpha (both p53-independent injury) were not. These results narrow the focus of our studies of the cellular pathways through which E1A

sensitizes tumor cells to immune-mediated apoptosis to mechanisms that are both p53-independent and resistant to blockade by Bcl-2 like antiapoptotic effects.

The results of our in vitro studies suggested that E1A expression should sensitize human breast cancer cells to host innate immune defenses (e.g., NK cells and activated macrophages) and to chemotherapy in vivo. We tested this prediction in collaborative studies with Dr. Jack Routes. In a previous collaboration, we observed that E1A expression eliminated the tumorigenicity of mouse fibrosarcoma cells in immunocompetent mice [9]. CD3 epsilon transgenic mice that lack killer lymphocyte function were, in contrast, equally susceptible to tumor formation by E1A-positive cells and E1A-negative cells. We used tumor induction in nude mice to test the independent effect of the innate immune responses on tumor development by E1A-expressing cells. Nude mice lack the ability to generate T-cell-dependent specific immune responses but retain the innate immune responses mediated by NK cells, activated macrophages and other T cell-independent host defenses. Nude mice exhibited and intermediate susceptibility to tumor formation by E1A-positive cells. Depletion of NK cells by pretreatment of nude mice with NK-binding antibodies resulted in significantly increased susceptibility to tumor formation by E1A-positive cells. These results indicated that the E1A-induce sensitivity to apoptosis triggered by NK cell-induced injury that we had observed in vitro is also involved in the in vivo tumor rejection process.

These mouse fibrosarcoma studies served as the basis for translational studies of our in vitro observations about E1A-induced apoptosis sensitivity of human breast cancer cells. For these experiments, we first characterized the in vitro effects of stable expression of the E1A gene in human breast carcinoma cells. These E1A-positive cells exhibited the expected increased susceptibility to in vitro killing by NK cells and activated macrophages (Fig. 1) and receptor-dependent triggering by Fas and TRAIL (Fig. 2). E1A-positive cells were also preferentially killed by diverse chemotherapeutic drugs, and this cell death was apoptotic in nature. The dose-response apoptosis triggering effect of the topoisomerase II inhibitor on E1A-positive breast cancer cells is shown in Figure 3A. The data in the bottom panel of Figure 3 shows that the etoposide-induced cell death of E1A-positive breast cancer cells is apoptotic, as evidenced by the increase in the percentage of apoptotic nuclei and DNA degradation in E1A-positive cells treated with etoposide. These data also showed that it was possible to repeat the essential in vitro parameters of tumor cell killing that had been identified for E1A-expressing cells from other species with human breast cancer cells.

We used these well-characterized cells to test the hypothesis that E1A expression would render human breast cancer cells more susceptible to rejection by the innate immune defenses of nude mice (Fig. 4). We did similar experiments with E1A-expressing human fibrosarcoma cells to determine whether observations with breast cancer cells were cell-type-specific (data not shown). The results of these experiments showed that E1A expression significantly prolonged the latency period for tumor development in nude mice (shift of tumor survival curve to the right; $p=0.04$) and reduced tumor inducing efficiency (shift upward in the tumor survival curve at the end point of evaluation). Nude

mice treated with a chemotherapeutic protocol exhibited a further prolongation in tumor latency and a reduction in tumor inducing efficiency by E1A-positive cells. The results of these experiments support the conclusion that expression of E1A oncoproteins in human breast carcinoma cells and in human fibrosarcoma cells renders the cells more susceptible to rejection by innate immune defenses and chemotherapy-induced rejection. Translated into the parlance of oncology studies of human tumors, these data suggest that E1A expression changes breast cancer cells in a way that would result in an increased "disease-free interval" and an increased sensitivity to chemotherapeutic interventions.

Discussion. Progress has been made in all areas of this proposal. We developed methods to test changes in cellular gene expression that are triggered by E1A and that correlate with E1A-induced increases in breast cancer cell sensitivity to immune-mediated and chemotherapy-induced injuries. We will use these new genetic data to expand our spectrum of experimental directions to further define mechanisms by which to convert highly resistant tumor cells into cells that are sensitive to a variety of host immunological defenses and therapeutic interventions. We have also begun to identify the cellular pathways through which these E1A-induced gene interactions control the tumor cell phenotype. These pathway studies will be important in structuring new hypotheses to test the molecular mechanisms by which cellular susceptibility to injury is controlled. The genetic and in vitro cytotoxicity data provided an experimental basis for translation of our cell biology studies into experimental tumor induction studies using human tumor cells. We developed quantitative methods for measuring changes in rates and efficiencies of tumor formation that are controlled by E1A expression. These experimental methods can be translated to other cell systems in which genetic modifications might alter tumor cell behavior in vivo.

KEY RESEARCH ACCOMPLISHMENTS:

- Translated in vitro observations made in an NIH-3T3 cell system to human breast cancer cells and human fibrosarcoma cells, which are also induced by E1A expression to exhibit increased susceptibility to a wide array of immunological and chemotherapeutic injuries.

- Determined that E1A-induced susceptibility of cells from different species and different tissue origins to cytolytic injury involves an apoptotic cell death response.

- Differential gene expression - identified E1A-related differences in cellular gene expression associated with increased tumor cell sensitivity to proapoptotic injuries – for use in hypothesis building regarding molecular mechanisms of E1A-induced apoptosis-sensitization.

- Cellular pathways - determined that E1A expression can induce sensitivity to proapoptotic injuries independently of cellular expression of the p53 tumor suppressor gene and of the antiapoptotic activities of the Bcl-2-like gene, E1B 19 kD.

- Translated in vitro observations regarding E1A-induced sensitivity of human tumor cells to immune-mediated and chemotherapy-induced apoptosis to an in vivo experimental tumor

system in nude mice and developed methods to quantitate E1A-related changes in tumor latency and tumor inducing efficiency in this system.

REPORTABLE OUTCOMES:

Manuscripts

1. Cook, JL, Routes, BA, Walker, T, Colvin, KL, and Routes, JM. E1A oncogene induction of susceptibility to killing by cytolytic lymphocytes through target cell sensitization to apoptotic injury. Experimental Cell Research 251: 414-423, 1999.
2. Cook, JL, Routes, BA, Leu, C, Walker, TA, and Colvin, KL. E1A oncogene-induced cellular sensitization to immune-mediated apoptosis is independent of p53 and resistant to blockade by E1B 19 kD protein. Exp. Cell Res. 252: 199-210, 1999.
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7. Cook, JL, Miura, T and Routes, JM. E1A-induced sensitivity of human tumor cells to innate immune defenses and chemotherapy-induced apoptosis: Correlations between in vitro cytolytic phenoptypes and quantitative changes in tumorigenicity. Submitted for publication, 2002.

Presentations:

1. Cook, JL, Colvin, KL, Routes, BA, Nichol, J, Worthen, GS and Walker, TA. E1A gene expression represses NF-kB-dependent cellular defenses against apoptosis. Imperial Cancer Research Fund DNA Tumor Viruses Meeting, 1997.
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7. Cook, JL, Miura, T and Routes, JM. E1A-induced sensitivity ot human tumor cells to innate immune defenses and chemotherapy-induced apoptosis: Correlations between in vitro cytolytic phenoptypes and quantitative changes in tumorigenicity. Molecular Biology of DNA Tumor Viruses Conference, 2002.

Development of Cell Lines and Animal Models:

1. E1A expressing MDA-MB435S cells
2. Nude mouse model of innate immune rejection and chemotherapy-induced rejection of oncogene-expressing human tumor cells – quantitation of tumor latency and tumor inducing efficiency.

CONCLUSIONS:

The results of these studies indicate that E1A-induced sensitization of neoplastic cells to both immune-mediated and chemically induced apoptosis is a common property of rodent cells and human breast cancer cells. The results show that phenotypic conversion of tumor cells from apoptosis-resistant to apoptosis-sensitive can be induced independently of expression of the p53 tumor suppressor gene and in a manner that is not blocked by antiapoptotic activity of Bcl-2 family members. Creation of E1A-positive human breast cancer cell lines provided the basis for studies of the molecular mechanisms of conversion to apoptosis-sensitivity and of the in vivo relevance of E1A-induced apoptosis sensitization for tumor rejection by innate immune defenses of nude mice and chemotherapy.

"So what?" The long-term goal of this project has been consistent throughout the period of funding – to use the adenoviral E1A oncogene as a tool to identify cellular pathways and molecular mechanisms that can be used to convert chemotherapy- and immunotherapy-resistant breast cancer cells into cells that are more sensitive to these therapeutic interventions. The results show important similarities between mouse cell models and human breast cancer cells that provide useful information for interpretation of studies from these two types of cell systems. The identification of specific cellular targets of E1A activity provide directions for future experiments to define the molecular triggers that can be used to sensitize tumor cells to therapeutic interventions.

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

1. James L. Cook, MD
2. Kelly L. Colvin
3. Vivian Zhang
4. Yong Kang

Appendix

Figure 1

E1A Expression Sensitizes Human Breast Cancer Cells to Killing by NK Cells and Activated Macrophages

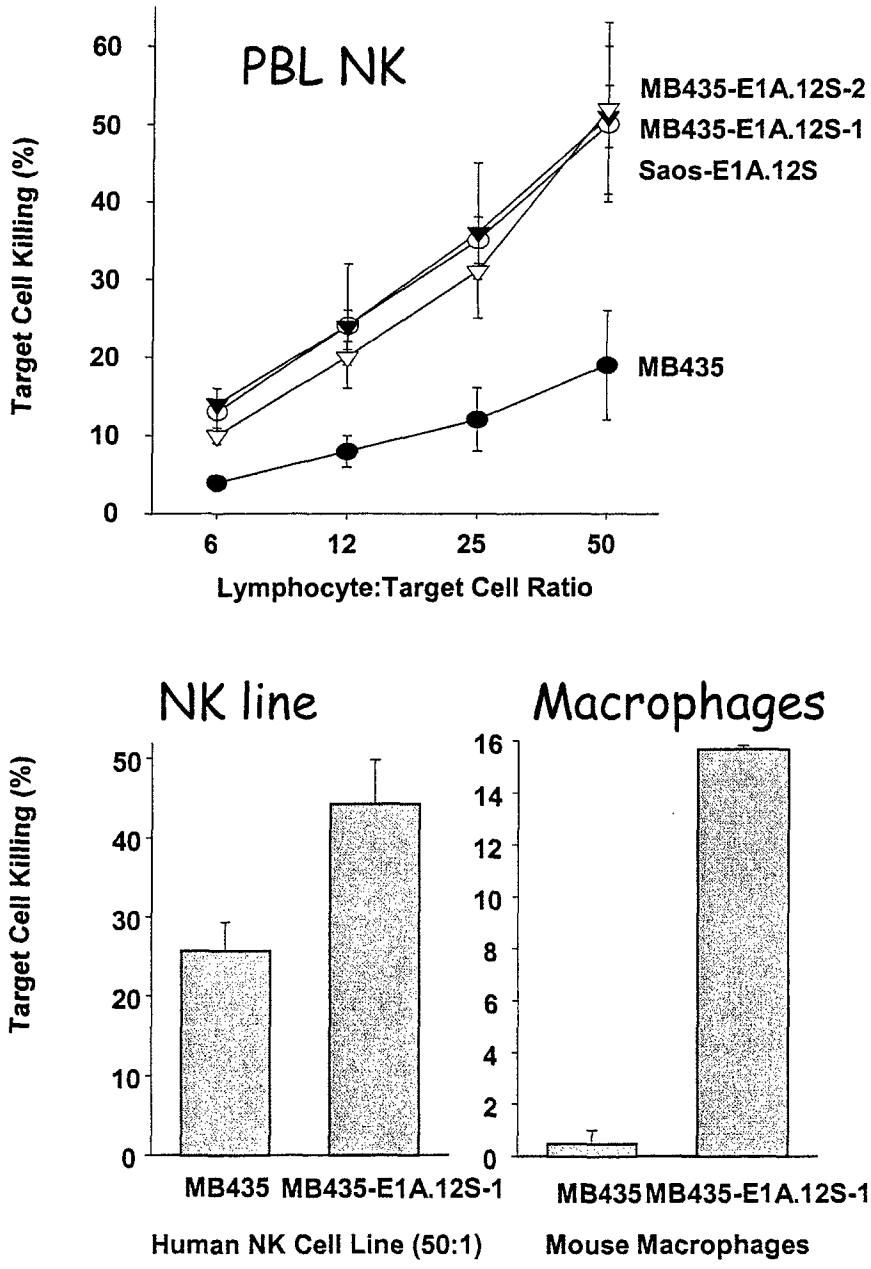


Figure 2

E1A Expression Sensitizes Human Breast Cancer Cells to Death Receptor-Triggered Apoptosis

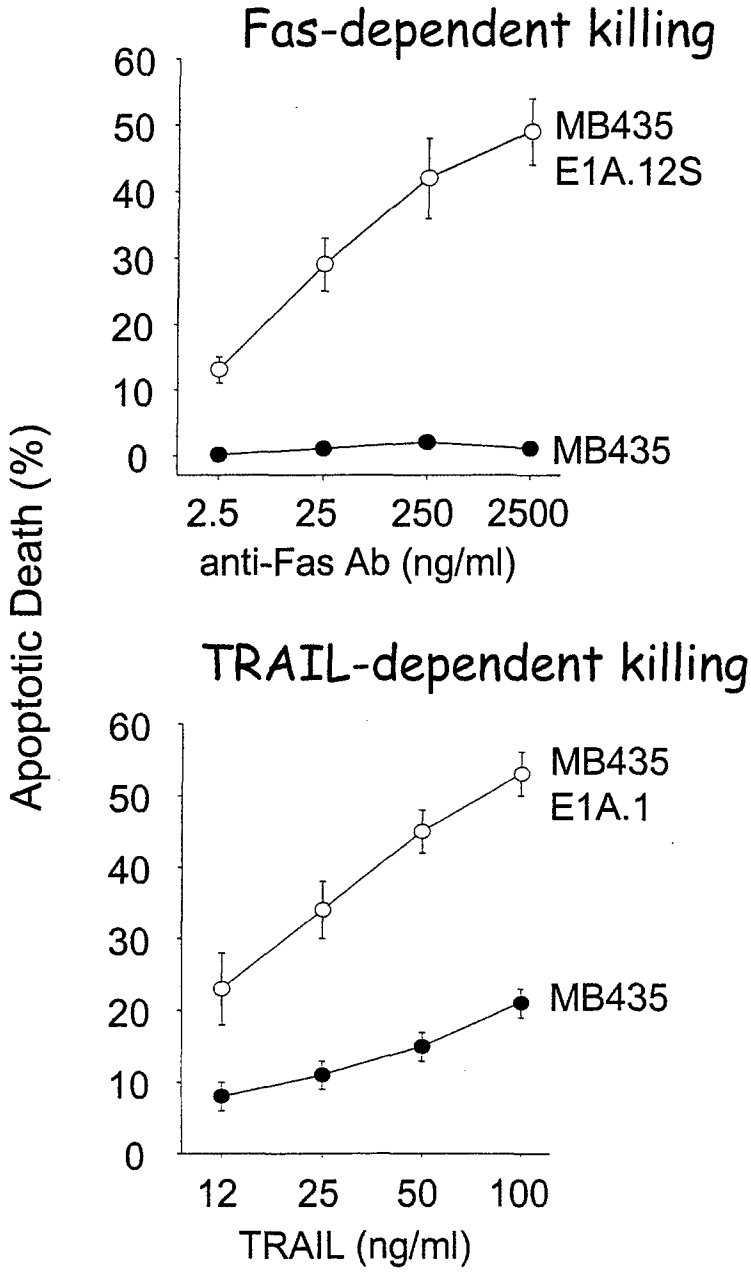


Figure 3

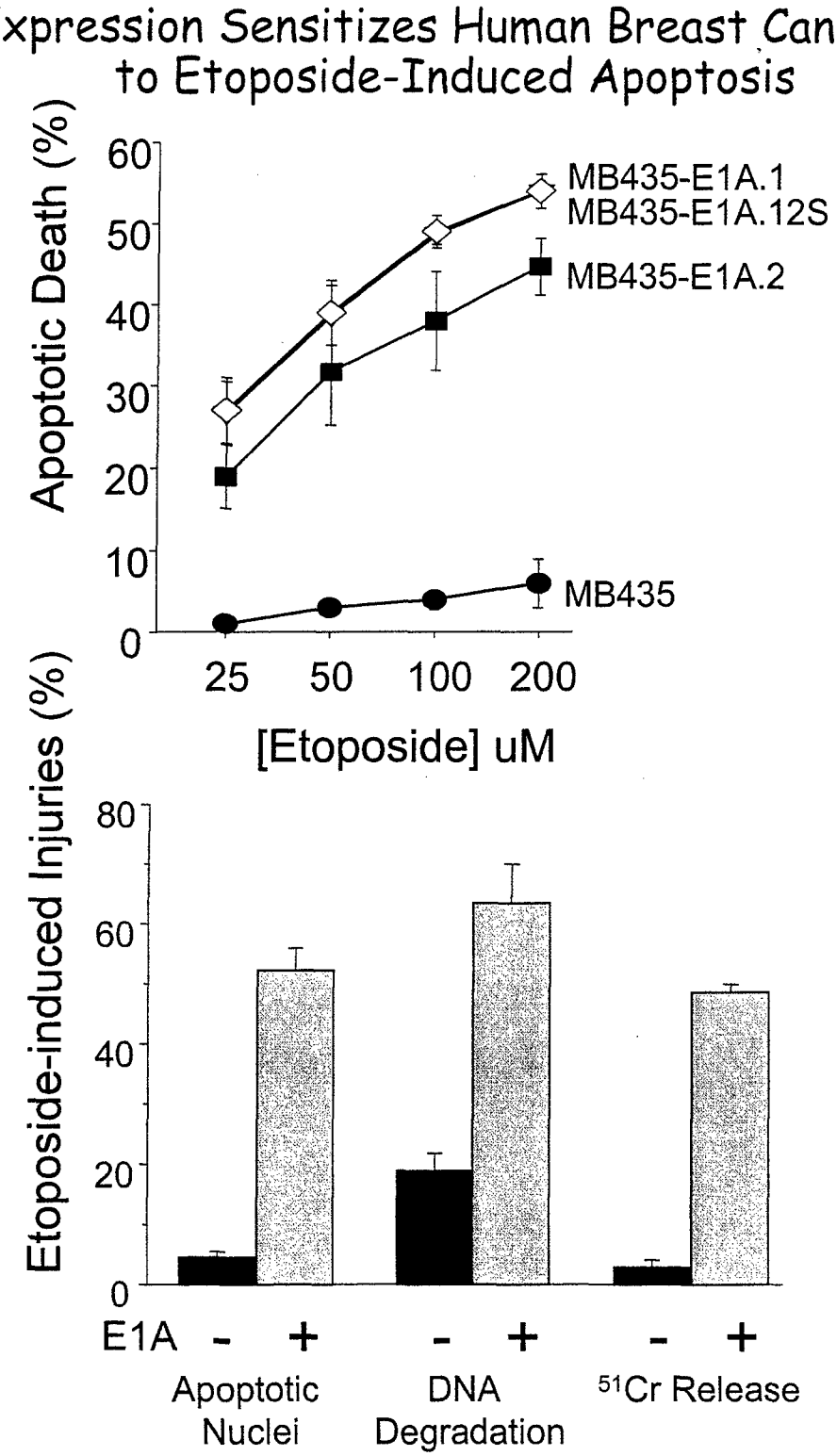
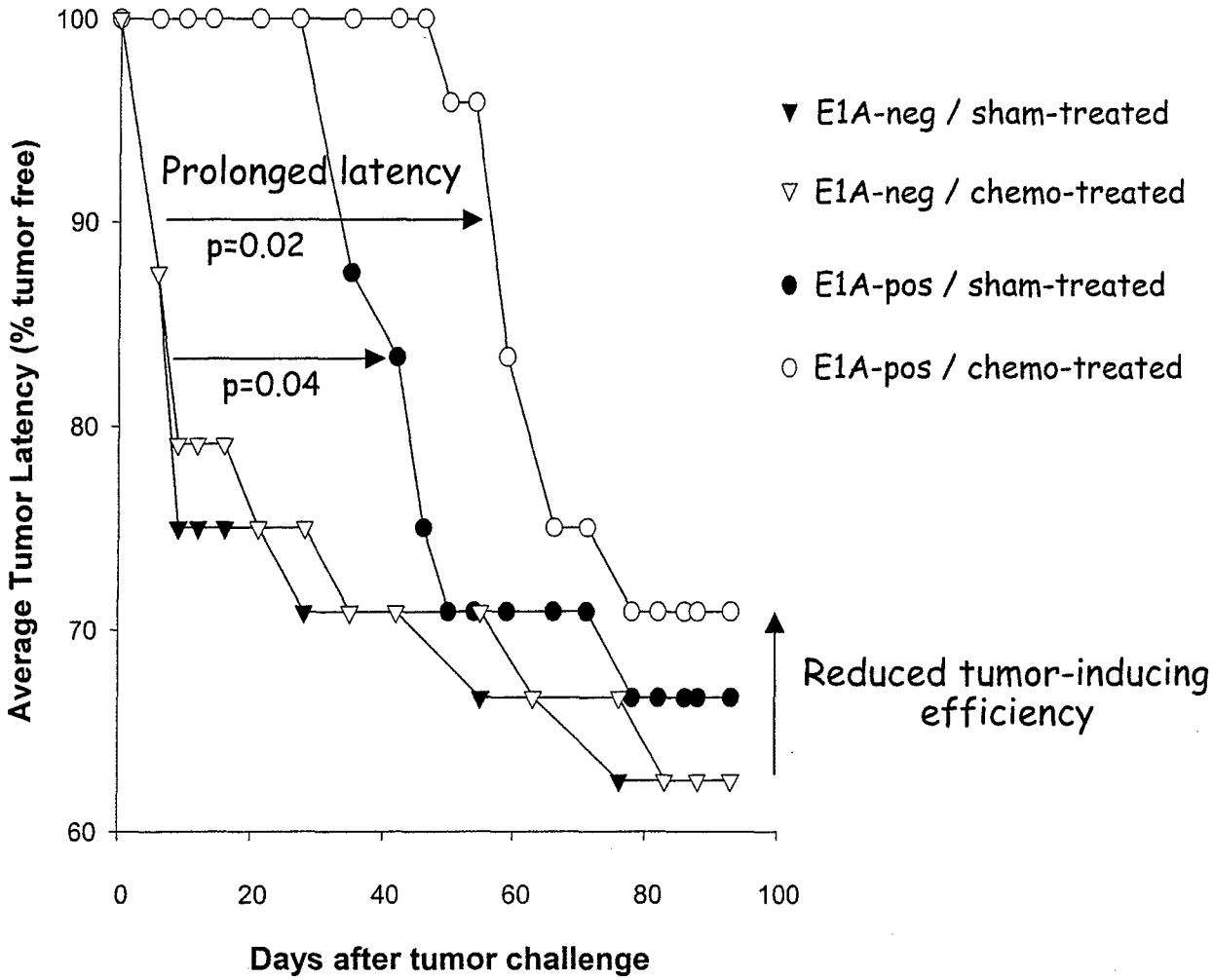

E1A Expression Sensitizes Human Breast Cancer Cells to Etoposide-Induced Apoptosis
Apoptotic Death (%)
60
50
40
30
20
10
0
MB435-E1A.1
MB435-E1A.12S
MB435-E1A.2
MB435
25
50
100
200
[Etoposide] uM
Etoposide-induced Injuries (%)
80
60
40
20
0
E1A - + - + - +
Apoptotic Nuclei
DNA Degradation
51Cr Release

Figure 4

E1A Expression Sensitizes Human Breast Cancer Cells to Nude Mouse Innate Immune Defenses and to Chemotherapy In Vivo



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